African Journal of Biotechnology Vol. 11(24), pp. 6450-6456, 22 March, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1626
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Agrobacterium-mediated transformation of watermelon (*Citrullus lanatus*)

Juan Li, Yi Tang, Yaoguo Qin, Xiaomei Li and Huanxiu Li*

College of Horticulture, Sichuan Agricultural University, Ya'an 625014, China.

Accepted 9 March, 2012

Transformation of watermelon (*Citrullus lanatus* cv. Zaojia) using *Agrobacterium tumefaciens* strain EHA105 containing the plasmid pRD400 carrying *Pti4* gene was studied in this work. Proximal cotyledons as explants were pre-cultivated for two day in the dark and it was found that the best condition for transformation of watermelon was the use of bacteria at a concentration of OD600 0.3, inoculation for 10 min and 72 h of co-cultivation. Kanamycin as a selective agent in different concentration (75, 100 and 125 mg/l) was tested. Transformed explants on medium supplemented with 100 mg/l kanamycin received a higher efficiency of shoots with transgenic DNA (9.17%). Using specific primer to *Pti4* gene, PCR product with a size of 680 bp was amplified when the genomic DNA extracted from the transformants were used as templates. A total of 148 plants with percentage 10.28% showed positive results using PCR analysis and subsequently all were regenerated into whole plants.

Key words: Watermelon (*Citrullus lanatus Thumb*), transgenic, *Agrobacterium tumefaciens*, *in vitro*.

INTRODUCTION

Watermelon (*Citrullus lanatus Thumb*.) is an important cucurbit crop species and it is a popular fruit that is native to Central Africa (Dane et al., 2003). Watermelon is an economically important crop and a valuable alternative source of water in desert areas, and its fruits are rich in carbohydrates, vitamins and minerals (Anonymous, 1992). The soluble fiber in watermelon may help to reduce cholesterol and risk of heart disease, a good source of fiber, which is important for keeping digestive track operating properly by preventing constipation, hemorrhoids and diverticular disease (Sultana and Bari, 2003). The nutritive value of seeds is due to their high oil and protein content (Oyenuga and Fetuga, 1975). Watermelon is susceptible to a number of fungal, bacterial and viral diseases requiring annual field rotation, frequent chemical sprays, and disease-resistant cultivars (Compton and Gray, 1999). In propagation the introduction of new characters into watermelon by means of genetic manipulation is of great potential value, specially the traits that would conquer resistance to diseases and pests. The success of genetic manipulation using those methods strongly depends on the presence of an efficient plant regeneration system (Tabei et al., 1993).

Recent advances in insertion of bacterial, fungal and virus resistance genes through Agrobacterium-mediated transformation would facilitate the development of new disease resistant genotypes without significantly altering the genetic composition, and have made it possible to improve the productivity and quality beyond the limitations of traditional/conventional breeding. Numerous science studies have been conducted for plant regeneration via organogenesis or somatic embryogenesis from cotyledons or immature embryos of watermelons (Tabei et al., 1993; Compton and Gray, 1993). Only a few reports for the watermelon transformation system have been achieved (Choi et al., 1994; Ellul et al., 2003; Compton et al., 2004). Therefore, this paper describes a transformation system for watermelon by co-culturing cotyledonary explants with Agrobacterium strain EHA105 containing the plasmid pRD400 carrying *Pti4* gene (*Pti4* gene is a gene which has a DNA binding domain and a nuclear localization sequence and it can encode related putative transcription factors). In addition, factors that affect Agrobacterium- mediated transformation rate, such as pre-cultivation time, inoculation time, co-cultivation period and concentration of kanamycin during selection

*Corresponding author. E-mail: hxli62@163.com.

of transgenic shoots were also tested.

MATERIALS AND METHODS

Explants preparation

Watermelon (*C. lanatus* cv. Zaojia) mature seeds were rinsed thoroughly for 30 min under running tap water. Seed coats were shucked and embryos were surface sterilized for 30 min by immersing in 75% ethanol (v/v). Surface sterilization was carried out with 0.1% mercury chloride ($HgCl_2$) (w/v) for 10 min with gentle shaking. Three successive washings with sterile distilled water were performed to remove the traces of $HgCl_2$.

The basal medium used for all the experiments was Murashige and Skoog, 1962 (MS) mineral formulation containing standard salts and vitamins, 30 g/l sucrose and 8 g/l agar. The pH was adjusted to 5.7 before adding agar and the media were autoclaved for 20 min at 121°C under 1.1 kg/cm^2 pressure. Sterilized seeds were cultured in continuous darkness for three days, and then put on 16 h photoperiod. Cotyledon explants were excised from six day-old seedlings and they were cut into proximal and distal halves. Proximal cotyledons were used as explants in the experiment. Cultures were incubated at 25±1°C at 1500 to 2000 lux cool white fluorescent light. Each experiment was repeated three times with 30 cotyledons per treatment in each experiment.

Agrobacterium tumefaciens strain EHA105 preparation

Agrobacterium strain EHA105 containing the plasmid pRD400, which has the resistance to streptomycin was used. Bacteria was cultivated in yeast extract peptone (YEP) solid medium (10 g/l peptone, 10 g/l yeast extract, 5 g/l sodium chloride and 15 g/l agar), containing kanamycin (50 mg/l) and streptomycin (50 mg/l), for about 24 h. After that, a single colony was transferred to a 100 ml Erlenmeyer, with 50 ml of YEP liquid medium supplemented with kanamycin (50 mg/l) and streptomycin (50 mg/l) and cultivated under 180 rpm, 27°C, until an optical density of 0.6 to 0.8 at 600 nm was reached. Bacteria suspension was centrifuged at 4800 rpm and resuspended in MS liquid medium until OD600 reached 0.3. The antibiotics were filter sterilized and added to the autoclaved medium.

Agrobacterium-mediated transformation methods

Before inoculation with Agrobacterium solution, explants of proximal cotyledons were pre-cultivated in MS medium supplemented with 2 mg/l benzyladenine (BA) for two days in darkness at 25°C. Infection and co-cultivation of explants with Agrobacterium were performed by soaking the explants in Agrobacterium suspension for 10 min and then blotting them dry, followed by incubation for two days in the dark at 27°C on MS medium supplemented with BA (2 mg/l). After co-cultivation, the explants were washed three times with sterile distilled water containing filter sterilized cefotaxime (500 mg/l), blotted dry and were subjected to selection. The explants were transferred to MS medium supplemented with BA (2 mg/ l), kanamycin (100 mg/l) and cefotaxime (500 mg/l) for testing transformed explants. Explants were subcultured every two weeks and two subcultures were done on the same medium. Well developed shoots were transferred to MS medium supplemented with BA (2 mg/l), kanamycin (100 mg/l) and cefotaxime (250 mg/l) for elongation and the roots were induced on half strength MS medium supplemented with IBA (0.3 mg/l), kanamycin (100 mg/l) and cefotaxime (250 mg/l). Plants that were still green after this selection period were then subcultured every two weeks and when roots were at least 4 cm long, the plantlets were transplanted under *ex vitro* condition into plastic pots containing autoclaved soil. All the pots were covered with clear plastic covers for seven days to maintain high humidity and incubated at 25°C under 16\ h photoperiod in a tissue culture chamber. Plantlets in the pots were watered every three days. After nine days, the humidity was reduced by progressively removing the lids after every three days. During this time, plants were watered two to three times alternatively with nutrient solution and water.

Different experiments were performed in order to evaluate the factors influencing the transformation efficiency, as follows: 1) proximal cotyledon explants excised from six-day-old seedlings were pre-cultured for 0, one, two and three days in darkness; 2) explants were infected with Agrobacterium suspension for 5, 10, 20 and 30 min; 3) explants were co-cultured with Agrobacterium suspension for two, three and four days period; 4) the shoots selection medium was supplemented with kanamycin at 75, 100 and 125 mg/l. All experiments had three replications per treatment. Each replication consisted of 30 explants. The experiments were repeated at least twice.

PCR of transgenic DNA

PCR amplification was used to confirm the presence of the T-DNA in kanamycin-resistant shoots. Genomic DNA was isolated from young leaves of both non-transformed plants and transformed kanamycin-resistant shoots as described by Akashi et al. (2004). The DNA was stored at -20°C. Specific primer was designed based on the nucleotide sequence of *Pti4* gene. The forward sequence of *Pti4* was 5' TCGGCTATGACTGGGCACA 3' and the reverse sequence was 5' GATACCGTAAAGCACGAGGAAG 3'. The designed primer was then used to amplify a fragment of *Pti4* gene with 680 bp. PCR amplification reaction was used in a final volume of 25 µl containing 10X PCR buffer (10 mM Tris-HCl, 25 mM $MgCl_2$ and pH 7.5), 1.5 mM dNTPs, 10 µM for each primer, 50 ng of template DNA and 0.5 U of *Taq* polymerase (TaKaRa , Japan). Reactions were performed in a thermocycler (Eppendorf, Germany). PCR conditions as follow: one cycle of 94°C for 4 min (denaturation), 35 cycles of 94°C for 30 s, 58°C for 30 s (annealing) and 72°C for 1 min (extension) and a final extension of 10 min at 72°C. PCR products were analyzed using 1% agarose gel electrophoresis. They were stained with 0.5 µg/ml ethidium bromide and visualized (probably in a trans-illuminator). The sizes of the fragments were estimated based on a DNA ladder of 2000 bp.

RESULTS AND DISCUSSION

The effect of pre-cultivation time

Different pre-cultivation period (0, one, two and three days) were tested to determine the effect to transgenic efficiency. Watermelon explants without pre-cultivation expanded more rapidly than pre-cultivated explants (Figure 1), subsequently affecting the transformation efficiency. There is hardly any literature about the procedures of pre-cultivation used in Agrobacterium-mediated transgenic processes. Some reports indicate that explants were inoculated with bacteria solution without any pre-treatments at all (Ibrahim et al., 2009; Akashi et al., 2005; Suratman et al., 2010; Youk et al., 2009; Mendes et al., 2002). A two-day pre-cultivation

Figure 1. *Citrullus lanatus* cv. Zaojia cotyledons without pre-cultivatio n

Table 1. The effect of pre-cultivation time to the transformation of *Citrullus lanatus* cv. Zaojia.

Pre-culture time/d	No. of total explant	Green kanamycin-resistant shoot	
		No.	Ratio (%)
0	90	0	0
1	90	3	3.33
2	90	8	8.89
3	90	4	4.44

gave the best results of 8.89% kanamycin resistant shoots followed by three-days (4.44.0%) and one day (3.33%).

Explants that were not pre-cultivated did not produce any kanamycin-resistant shoots (Table 1). Result under discussion related to the differentiation state of cotyledon cells, competent cells of explants were in active state and in higher differentiation rate only if they were pre-cultivated for a proper time. However, pre-cultivating for two days in this work was the best time, as well as competent cells of explants were easy to integrate with Agrobacterium and more green resistant buds were obtained.

The effect of Agrobacterium inoculation time

The effect of inoculation time was further investigated. The overall results obtained for this experiment are summarized in Table 2. Generally, the frequency of kanamycin-resistant shoots increased with the increase of inoculation time. However, according to our result the longer the inoculation time was, the lower the frequency of kanamycin-resistant buds was, which were in disagreement to few reports published (Suratman et al., 2010, Park et al., 2005; Padmanabhan and Sahi, 2009). The highest percentage of kanamycin-resistant shoots was obtained at 10 min (10%). Inoculation longer than 10 min did not increase the transformation efficiently. Therefore, 10 min was found to be closer to the optimum inoculation period and was therefore selected for subsequent experiment Inoculation time of the explants with the Agrobacterium plays an important role in gene transfer. The time will determine the most effective condition for maximum gene transfer to take place. In the present study, the highest percentage of kanamycin-resistant buds was obtained at 10 min of inoculation. This contradicts Silva et al. (2009) who found twenty minutes inoculation to be the optimum for transformation of

Table 2. The effect of Agrobacterium inoculation time to the transformation of *Citrullus lanatus* cv. Zaojia.

Infection time/min	No. of total explant	Green Kanamycin-resistant shoot	
		No.	Ratio (%)
5	90	4	4.44
10	90	9	10.00
20	90	7	7.78
30	90	5	5.56

Table 3. The effect of co-cultivation period to transformation of *Citrullus lanatus* cv. Zaojia.

Co-cultivation period/h	No. of total explant	Green kanamycin- resistant shoot	
		No.	Ratio (%)
24	90	2	2.22
48	90	6	6.67
72	90	10	11.11
96	90	3	3.33

Table 4. The effect of different concentration of kanamycin to the selection of *Citrullus lanatus* cv. Zaojia transgenic shoots.

Concentration of kannmycin (mg/l)	No. of total explant	Inductivity (%)	Green kanamycin resistant shoot		Shoots with transgenic DNA by PCR	
			No.	Ratio/%	No.	Ratio/%
75	120	63.3	20	16.67	8	6.67
100	120	35.8	12	10.00	11	9.17
125	120	23.3	7	5.83	7	5.83

Theobroma cacao.

The effect of co-cultivation period

Transformation experiments were performed to optimize the effective *A. tumefaciens* co-cultivation period for transformation. In the present study, co-cultivation period was assessed for each hour from 24 to 96 h. It was observed that simple inoculation of the explants with Agrobacterium and its transfer to selection medium did not allow the regeneration of transgenic plants as also demonstrated by Mendes et al. (2002) in 'Hamlin' sweet orange. 72-h co-cultivation was found optimal for zaojia watermelon as it obtained the production of significantly higher rate of kanamycin-resistant buds (11.11%) as compared to other hours of co-cultivation (Table 3). It was clear that 72 h was possible to produce the highest gene expression for cotyledon explants than other treatments. The findings are in agreement with Ibrahim et al. (2009) who found co-cultivation for 72 h with *A. tumefaciens* gave the highest number and percentage of transformation frequency in watermelon.

The effect of different concentration of kanamycin to the selection of transgenic shoots

Kanamycin resistance is the most widely used selectable marker for plant transformation and the sensitivity of a particular species or explant to kanamycin is a key element in the establishment of any new transformation system in which a kanamycin resistance gene will be employed.

To establish a method for selection of transgenic plants the inoculated explants were cultivated on various concentrations of kanamycin, and the efficiency of shoot induction was examined four weeks after inoculation (Table 4). In our study, shoot induction was highest on the medium containing the lowest concentration of kanamycin of 75 mg/l (20 shoots), decreased as kanamycin concentration was increased to 100 mg/l (12 shoots) and further decreased at 125 mg/l (7 shoots) (Table 4). However, PCR assay of the leaves harvested from these shoots showed that 100 mg/l kanamycin produced the highest number of transgenic shoots (11 out of 12 shoots) compared to 75mg/l and 125 mg/l kanamycin concentration at this concentration which produced an average

Figure 2. *Citrullus lanatus* cv. Zaojia untransformed shoots on medium containing kanamycin.

Figure 3. *Citrullus lanatus* cv. Zaojia transformed shoots with T-DNA.

of eight and seven transgenic shoots respectively (Table 4). These results indicate that some shoots failed to integrate the foreign gene but was bleached on the selected medium containing kanamycin (Figure 2). Ibrahim et al. (2009) study showed that no survived (100% mortality) explants were observed when 100 mg/l kanamycin was used in all types of explants. These results are in line with Raharjo et al. (1996) who found that kanamycin-resistant embryogenic calli were used to initiate suspension cultures (in liquid MS medium with 1.0 µM 2,4-dichlorophenoxyacetic acid (2,4-D)/BA, 50 mg/l kanamycin) for multiplication of embryogenic cell aggregates. Joung et al. (2001) reported that kanamycin was used for the selection of putative transformants. The minimum concentration of kanamycin required to kill non-transformed leaf explants of Campanula was 50 mg/l, and death of explants occurred after three months of culture.

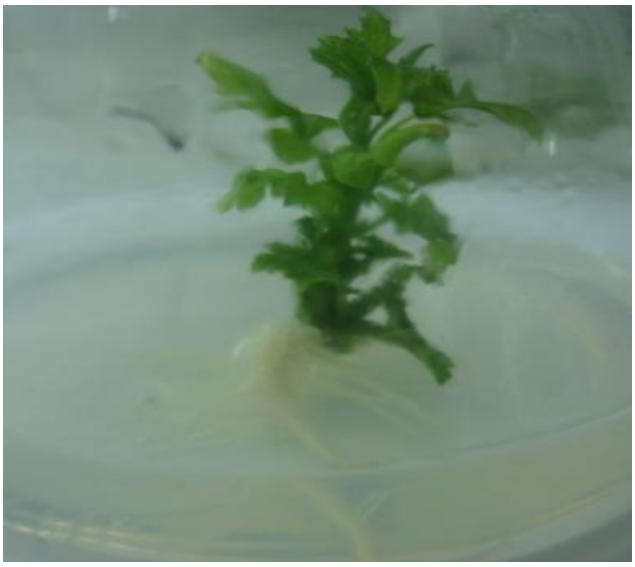

Figure 4. *Citrullus lanatus* cv. Zaojia transgenic plantlets.

PCR analysis

The PCR is a sensitive technique allowing single-copy genes to be amplified and extracted out of a complex mixture of genomic sequence (Innis and Gelfand, 1990). PCR was utilized in this investigation for rapid screening of *Pti4* gene explants. Total genomic DNA was isolated from explants of putative transformants. DNA of non-transformed explants was used as a negative control and DNA of pRD400 (isolated from Agrobacterium strain EHA105) was used as a positive control as shown in Figure 5. A designed primer specific to *Pti4* gene was used for PCR amplification and a PCR product with a size of about 680 bp was amplified. The genomic DNA of the transformants (Figure 3) was used as templates (lanes 1 to 15). The obtained results confirm the presence of *Pti4* in the transformants. Amplification of this fragment (680 bp) was not observed in untransformed plants (lane CK1). Around 148 (10.28%) of the plants showed positive results using PCR analysis. It is laborious and time consuming to screen for 1440 multiple shoots. The 10.28% positive PCR does not assure that they are truly positive transgenic as chimeric shoots could also show positive results in PCR. Furthermore, all of the transformants regenerated into whole plant (Figure 4). Therefore, due to the difficulty associated with

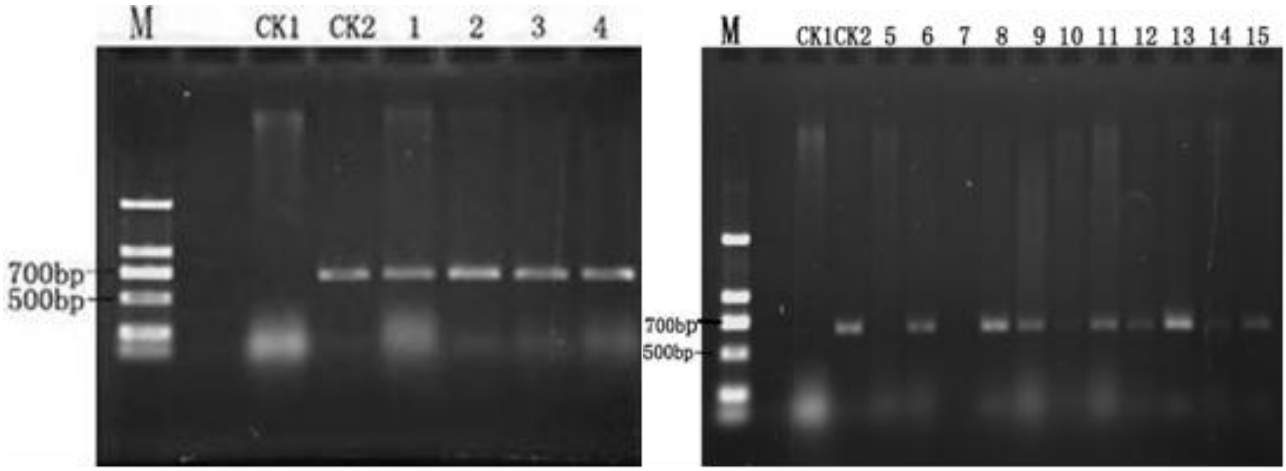

Figure 5. PCR analysis in Km resistance *Citrullus lanatus* cv. Zaojia plants. CK1, Negative contrast; CK2, positive contrast; 1 to 15, the number of Km resistance plants.

selection using hygromycin and kanamycin, it is proposed that an alternative selection system, such as the use of herbicide Basta (active ingredient PPT or phosphinothricin acetyl transferase), be evaluated for this yellow watermelon in the future. Another alternative to the difficulty in regenerating transformed multiple shoots is to transform the plant without using selectable marker gene as reported for potato (De Vetten et al., 2003). Furthermore the transformed watermelon was difficult to develop further after subculture. In every situation, explants suffered from intense enzymatic browning followed by necrosis and eventually die. Similar observation on the browning and dying of multiple shoots was also reported on *Impatiens walleriana* when multiples shoots were selected on kanamycin (Baxter, 2005). Ezura et al. (2000) also reported that ethylene production was promoted by *A. tumefaciens* inoculation and the increased levels of ethylene in the culture vessel or flasks of transformed melon cotyledon resulted in a reduction in the efficiency of gene transfer and growth.

Conclusion

In this study, we successfully produced transgenic watermelon using *A. tumefaciens* after optimizing a couple of transformation parameters. Proximal cotyledons as explants were pre-cultivated for two days in the dark and it was found that the best condition for transformation of watermelon was using bacteria at a concentration of OD600 0.3, inoculation for 10 min and 72 h of co-cultivation. Kanamycin as a selective agent in different concentration (75, 100 and125 mg/l) was tested. Transformed explants on medium supplemented with 100 mg/l kanamycin received a higher efficiency of shoots with transgenic DNA (9.17%). Using specific primer to *Pti4* gene, PCR product with a size of 680 bp was amplified when the genomic DNA extracted from the transformants were used as templates. A total of 148 plants with percentage 10.28% showed positive results using PCR analysis and subsequently all were regenerated into whole plants.

ACKNOWLEDGEMENTS

This work was supported by Science and Technology Department of Sichuan Province (09zc270) and Shuangzhi plan of Sichuan Agricultural University Fund.

REFERENCES

Akashi K, Nishimura N, Ishida Y, Yokota A (2004). nPotent hydroxyl radical-scavenging activity of drought-induced type-2 metallothionein in wide watermelon. Biothem. Biophy. Res. Commun. 323: 72-78.

Akashi K, Morikawa K, Yokota A (2005). Agrobacterium-mediated transformation system for the drought and excess light stress-tolerant wild watermelon *(Citrullus lanatus)*. Plant Biotechnol. 22(1): 13-18.

Anonymous (1992). In: The wealth of India-a dictionary of Indian raw materials and industrial products, Ca-Ci. New Delhi: Publication and Information Directorate, CSIR; 3: 606-609.

Baxter A (2005). Regeneration and transformation of Impatiens walleriana using cotyledonary node culture. M.Sc. Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Choi PS, Soh WY, Kim YS, Yoo OJ, Liu JR (1994).Genetic transformation and plant regeneration of watermelon using *Agrobacterium tumafaciens*. Plant Cell Rep. 13: 344-348.

Compton ME, Gray DJ (1993). Somatic embryogenesis and plant regeneration from immature cotyledons of watermelon. Plant Cell Rep. 12: 61-65.

Compton ME, Gray DJ (1999). Shoot organogenesis from cotyledon explants of watermelon. In: Trigiano RN, Gray DJ, eds. Plant tissue culture concepts and laboratory exercises. 2nd edn. Boca Raton, FL: CRC.Press; 149-157.

Compton ME, Gray DJ, Gaba VP (2004). Use of tissue culture and biotechnology for the genetic improvement of watermelon. Plant Cell. Tissue Organ Cult. 77: 231-243.

De-Vetten N, Walters A, Raemakers K, Der-Meer IV, Ter-Stege R (2003). A transformation method for obtaining marker-free plants of

across-pollinating and vegetatively propagated crop. Nat. Biotechnol. 21: 439-442.
Ellul P, Rios G, Atares A, Roig LA, Serrano R, Moreno V (2003).The expression of the Saccharomyces cerevisiae HAL1 gene increases salt tolerance in transgenic watermelon [*Citrullus lanatus* (Thunb.) Matsun. & Nakai.]. Theor. Appl. Genet.107: 462-469.
Ezura H, Yuhashi KI, Yasuta T, Minamisawa K (2000). Effect of ethylene on Agrobacterium tumefaciens-mediated gene transfer to melon. Plant Breed. 119: 75-79.
Ibrahim IA, Nower AA, Badr-Elden AM, Abd Elaziem TM (2009). High Efficiency Plant Regeneration and Transformation of Watermelon (*Citrulus lanatus c*v. Giza 1). Res. J. Agric. Biol. Sci. 5(5): 689-697.
Innis MA, Gelfand DH (1990). Optimization of PCRs. In PCR protocols: A guide to methods and application (eds. Innis MA, Gelfand DH, Sninsky JJ and Thomas TJ). Academic Press, San Diego. pp. 3-12.
Joung YH, Roh MS, Kamo K, Song JS (2001). Agrobacterium-mediated transformation of Campanula glomerata. Plant Cell Rep. 20: 289-295.
Mendes BMJ, Boscariol RL, Filho FAAM, Almeida WAB (2002). Agrobacterium-mediated genetic transformation of 'Hamlin' sweet orange. Pesq. Agropec. Bras. 37(7): 955-961.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant.15: 473-497.
Oyenuga VA, Fetuga BL (1975). Some aspects of the biochemistry and nutritive value of the watermelon seed (*Citrullus vulgaris* schrad). J. Sci. Food Agric. 26: 843-846.
Padmanabhan P, Sahi SV (2009). Genetic transformation and regeneration of *Sesbania drummondii* using cotyledonary nodes. Plant Cell (watermelon). Biotechnol. Rep. 8: 393-404. Rep. 28: 31-40.
Park SM, Lee JS, Jegal S, Jeon BY, Jung M (2005). Transgenic watermelon rootstock resistant to CGMMV (cucumber green mottle mosaic virus) infection. Plant Cell Rep. 24: 350-356.
Raharjo SHT, Hernandez MO, Zhang YY, Punja ZK (1996). Transformation of pickling cucumber with chitinase-encoding genes using Agrobacterium tumefacienss. Plant Cell Rep. 15(8): 591-596.
Silva TER, Cidade LC, Alvim FC, Cascardo JCM, Costa MGC (2009). Studies on genetic transformation of *Theobroma cacao* L.: Evaluation of different polyamines and antibiotics on somatic embryogenesis and the efficiency of uidA gene transfer by *Agrobacterium tumefacienss*. Plant Cell. Tissue Organ Cult. 99: 287-298.
Sultana RS, Bari MA (2003). Effect of different plant growth regulators on direct regeneration of watermelon (*Citrulus lanatus* Thumb). Plant Tissue Cult. 13(2): 173-177.
Suratman F, Huyop F, Wagiran A, Rahmat Z, Ghazali H, Parveez GKA (2010). Cotyledon with Hypocotyl Segment as an Explant for the Production of Transgenic Citrullus vulgaris Schrad (Watermelon) Mediated by *Agrobacterium tumefacienss*. Biotechnol. 9(2): 106-118.
Tabei Y, Yamanaka H, Kanno T (1993). Adventitious shoot induction and plant regeneration from cotyledons of mature seed in watermelon (*Citrullus lanatus* L.). Plant Tissue Culture Lett. 10: 235-241
Youk ES, Pack IS, Kim YJ (2009). A framework for molecular genetic assessment of a transgenic watermelon rootstock line. Plant Sci. 176: 805-811.